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ARTICLE

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The effects of hydration and divalent cations on lamellar-nonlamellar phase transitions in membranes and total lipid extracts from *Acholeplasma laidlawii* A-EF22 – a ^{2}H NMR study

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Abstract *Acholeplasma laidlawii* strain A-EF22 was grown in a medium supplemented with 75 μM α-deuterated palmitic acid (16:0-d_2) and 75 μM α-deuterated oleic acid (18:1c-d_2), or with 150 μM 18:1c-d_2. The fatty acids were incorporated into the membrane lipids and ^{2}H NMR spectra were recorded from intact membranes, total lipid extracts, and the combined glucolipid and neutral lipid fractions of a total lipid extract. The lipids in intact membranes form a bilayer structure up to at least 70 °C. The same result was obtained with membranes digested with pronase, which removes a large fraction of the membrane proteins. A reversed hexagonal liquid crystalline (H_{II}) phase was formed below 70 °C by the total lipid extracts hydrated with 20 and 30% (w/w) water; in the presence of 40% (w/w) water only one of the extracts formed an H_{II} phase below 70 °C. The H_{II} phase was formed at higher temperatures with an increasing water content. However, only a lamellar liquid crystalline (L_α) phase was formed up to 70 °C by the total lipid extracts when the water concentrations were 50% (w/w) or higher. The temperature (T_{LH}) for the L_α to H_{II} phase transition in the combined glucolipid and neutral lipid fractions was only 2–3 °C lower than for the total lipids, and the phospholipids thus have a very modest influence on the T_{LH} value. Physiologically relevant concentrations of Ca^{2+} and Mg^{2+} ions did not affect the phase equilibria of total lipid extracts significantly. It is concluded from comparison with published data that the membrane lipids of the cell wall-less bacterium *A. laidlawii* have a smaller tendency to form reversed nonlamellar phases than the membrane lipids of three bacterial species surrounded by a cell wall.

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1 Introduction

Practically all biological membranes contain at least one lipid that is able to form a reversed nonlamellar liquid crystalline phase, such as a reversed hexagonal (H_{II}) phase or a reversed cubic (I_{II}) phase. Data accumulated since the beginning of the sixties show that total lipid extracts, isolated from membranes of prokaryotic as well as eukaryotic origin, are also able to form reversed nonlamellar phases (Luzzati and Husson 1962; Gulik-Krzywicki et al. 1967; Rivas and Luzzati 1969; Burnell et al. 1980; Gulik et al. 1985; Lindblom et al. 1986; Mariani et al. 1990; Morein et al. 1996). It has even been suggested that the lipids in intact membranes may form nonbilayer structures (Burnell et al. 1980; De Kruijff et al. 1980).

The phase behaviour of lipid-water systems is influenced by a number of factors. An increase in the temperature, and a decrease in the water content, often favour the formation of H_{II} and I_{II} phases (Rilfors et al. 1984; Lindblom and Rilfors 1989; Seddon 1990; Lindblom et al. 1991). Accordingly, some total lipid extracts, like those prepared from human brain, beef heart mitochondria and maize chloroplasts, form an H_{II} phase at water contents below approximately 20% (w/w), but form a lamellar liquid crystalline (L_α) phase at higher water contents (Luzzati and Husson 1962; Gulik-Krzywicki et al. 1967; Rivas and Luzzati 1969). On the other hand, total lipid extracts isolated from *Escherichia coli* membranes form H_{II} and/or I_{II} phases with water contents between 20 and 95% (w/w) (Burnell et al. 1980; Ranck et al. 1984; Killian et al. 1992; Morein et al. 1996). *E. coli* cells are surrounded by two membranes and a cell wall. In contrast, cells of *Acholeplasma laidlawii* have no cell wall and are surrounded by a single membrane. Total lipid extracts prepared from *A. laidlawii* A-EF22 membranes can form H_{II} and/or I_{II} phases with approximately 10 and 20% (w/w) water (Lind-

blom et al. 1986; Rilfors et al. 1994; Österberg et al. 1995), i. e. at water contents roughly corresponding to the maximum hydration of the different glucolipids (Lindblom et al. 1986, 1993). However, no studies of the phase equilibria of total *A. laidlawii* lipid extracts at higher water contents have been performed so far. The present work fills this gap and the results are compared with those obtained with total lipid extracts prepared from *E. coli* and other cell wall-surrounded bacteria.

Anionic phospho- and phosphoglucolipids constitute approximately 15–45 mol% of the membrane lipids in *A. laidlawii* A-EF22 (Wieslander et al. 1980, 1995; Andersson et al. 1996). Reversed nonlamellar phases are formed by some anionic phospholipids in the presence of divalent cations (Lindblom et al. 1991, and references therein). Therefore, the effect of physiologically relevant concentrations of Ca^{2+} and Mg^{2+} ions on the phase equilibria of total lipid extracts are also reported in the present study.

2 Materials and methods

2.1 Cell growth

A. laidlawii strain A-EF22 was grown in a medium (Eriksson et al. 1991) containing lipid-depleted bovine serum albumin, fraction V (Boehringer Mannheim GmbH, Mannheim, Germany) (Razin and Rottem 1976) and lipid-depleted tryptose (Difco Laboratories, Detroit, MI), prepared as described by Niemi et al. (1995). The medium was supplemented with 75 μM α-deuterated palmitic acid (16:0-d_2) and 75 μM α-deuterated oleic acid (18:1c-d_2), or with 150 μM 18:1c-d_2. The deuterated fatty acids were synthesized according to Tulloch (1977) and the purity of these acids was 99% as determined by gas-liquid chromatography (GLC). *A. laidlawii* cells from 11 of medium were used for preparation of the membrane samples, and cells from 5 or 30 l of medium were used for preparation of the total membrane lipid extracts. The cells were grown for 20 h at 30 °C.

2.2 Harvest of cells

The cell suspensions were centrifuged in a Beckman JA-10 rotor for 20 min at 17 600 g_{max}. Cells intended for preparation of total lipid extracts were washed in β-buffer (150 mM NaCl; 50 mM Tris; 0.2% (v/v) β-mercaptoethanol; pH = 7.4), centrifuged and frozen at –40 °C. Cells intended for preparation of intact membranes were washed in β-buffer supplemented with 10 mM Na_2-EDTA, centrifuged, and lysed in deionised water at 20 °C for 45 min under gentle stirring; after cell lysis Na_2-EDTA was added to a final concentration of 10 mM. The membrane suspensions were centrifuged at 1 °C in a Beckman JA-20 rotor for 45 min at 48 300 g_{max}. Finally, the membranes were washed in diluted β-buffer (1/20) and centrifuged.

In order to obtain membranes with a reduced amount of protein, a membrane pellet, from the cells grown in medium supplemented with 150 μM 18:1c-d_2, was resuspended in β-buffer and the proteolytic enzyme pronase was added to a final concentration of 250 μg/ml. The digestion was carried out at 40 °C for 30 min. The membrane preparation was centrifuged at 5 °C in a 70 Ti rotor for 30 min at 100 000 g_{max}. According to Morowitz et al. (1969) this proteolytic procedure removes more than 70% of the membrane proteins.

2.3 Preparation of membrane samples for ^{2}H NMR studies

The intact and the pronase-treated membrane samples were washed once in 4 ml of deuterium-depleted water (^1H_2O) (Fluka Chemika) to remove most of the deuterium signal from the buffer. The membrane samples were concentrated by ultracentrifugation at 1 °C in a TLA-100.3 rotor for 60 min at 202 000 g_{max}, and the pellets were transferred to 10-mm outer diameter (o. d.) NMR glass tubes.

In order to record a ^{2}H NMR spectrum of acceptable quality from the membrane samples, free induction decays (FIDs) were often accumulated during 2–3 h. A possibility therefore exists that membrane-associated enzymes may alter the lipid composition in the preparations while the spectra are recorded, and such alterations could in turn affect a possible transition between L_α and I_{II} or H_{II} phases. This possibility was excluded by inactivating the enzymes; the intact membrane samples, prepared from the cells grown in medium supplemented with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2, were heated to 70 °C for 10 min and then cooled on ice for 10 min. However, the inactivation of the membrane proteins per se may affect the phase equilibria of the membrane lipids, and the intact and the pronase-treated membrane samples, prepared from the cells grown in medium supplemented with 150 μM 18:1c-d_2, were not exposed to 70 °C before the spectra were recorded. The recording of the ^{2}H NMR spectra were initiated immediately after the membrane samples had been prepared.

2.4 Isolation and purification of lipids

The extraction of the lipids, and the purification of the lipid extracts on a Sephadex G-25 Fine column, were performed as described by Eriksson et al. (1991).

The isolation of the combined glucolipid and neutral lipid fractions from the total lipid extract containing 16:0-d_2 and 18:1c-d_2 was performed on a silicic acid column (Bio-Sil HA minus 325 mesh, Bio-Rad Laboratories, Richmond CA) (Eriksson et al. 1991). These lipid fractions were eluted with chloroform : methanol, 8 : 2 (v/v).

To remove the divalent cations, which can affect the phase equilibria of anionic membrane lipids (Lindblom et al. 1991, and references therein) the total lipid extracts, from the cells grown in medium supplemented with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2, were treated with Na_2-EDTA

in order to remove these ions. The total lipid extracts, from the cells grown in medium supplemented with 150 μM 18:1c-d_2, were treated with Na_2-EGTA, according to a modified version (Rilfors et al. 1994) of the procedure described by Smaal et al. (1985).

2.5 Determination of polar head group and acyl chain compositions

The medium supplemented with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2 also contained 3 μCi/l ^{3}H-16:0 and 1 μCi/l ^{14}C-18:1c. The glucolipid composition in the total lipid extract derived from cells grown in this medium was determined by a combination of TLC and liquid scintillation counting. The glucolipids were separated on DC-Alufolien Kieselgel 60 plates (Merck, Darmstadt, Germany) developed in chloroform : methanol : 25% ammonia, 91 : 35 : 10 (v/v). The lipids were detected and analysed as described by Niemi et al. (1995). The polar head group compositions of the total lipid extracts derived from cells grown in the medium supplemented with 18:1c-d_2 were determined by high performance liquid chromatography (HPLC), using a modified version (Andersson et al. 1996) of the method described by Arnoldsson and Kaufmann (1994). The peaks in the chromatograms were assigned by comparing their retention times with those of the purified *A. laidlawii* lipids and the molar percentages were obtained with the appropriate molar response factors (Andersson et al. 1996).

The acyl chain compositions in the different total lipid extracts were determined by GLC after converting the lipid acyl chains to their methyl esters (Rilfors et al. 1978). The analyses of the acyl chain composition for the total lipid extract derived from cells grown in the medium supplemented with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2 were performed with a Carlo Erba Instrument, Model HRGC 5300-HT, equipped with a 15 m capillary, fused silica column coated with Supelco Wax (Supelco Inc., Bellefont, PA). The analyses of the lipid extracts obtained from cells grown in medium supplemented with 150 μM 18:1c-d_2 were performed with a Varian Instrument, Model 3700, equipped with a 180 cm polar SP-2330 column (inner diameter 0.13 cm) from Supelco Inc. (Bellefonte, PA). Integrators were connected to both apparatuses. The acyl chain compositions were calculated after the response factors had been obtained from standard reference mixtures (Larodan Fine Chemicals, Malmö, Sweden).

2.6 Preparation of lipid samples for ^{2}H NMR studies

The lipid extracts were dried in 8 mm o. d. glass tubes under a stream of N_2, followed by freeze-drying at ~10^{-3} Torr until constant weights were obtained. The total lipid extracts were hydrated with 20, 30, 40, 50 or 60% (w/w) ^1H_2O and the combined glucolipid and neutral lipid fractions were hydrated with 20% (w/w) ^1H_2O.

To study the effect of divalent cations, two total lipid extract samples (derived from cells grown in the medium supplemented with 150 μM 18:1c-d_2, batch 2) were hydrated with 20% (w/w) ^1H_2O containing 134 mM $CaCl_2$ or $MgCl_2$, which corresponds to a molar ratio cations : anionic phospholipids of 1/10. The samples were freeze-thawed and centrifuged several times in order to mix all the components thoroughly.

2.7 ^{2}H NMR measurements

^{2}H NMR spectra were recorded with a Bruker AMX2-500 spectrometer operating in the quadrature detection mode at the frequency 76.77 MHz using a standard broad-band high resolution probe. The temperature was controlled with a Bruker BV-T unit, calibrated before each temperature scan. The sample was allowed to equilibrate for 30 min at each temperature before the measurements were made. A phase-cycled quadrupole echo pulse sequence was used (Bloom et al. 1980) with a $\pi/2$-pulse width of 15 μs and a delay time, between the two pulses, of 60 μs. For each sample and temperature, ~45 000 FIDs were accumulated with a recycle time of 150 ms. The spectral width was 167 kHz. All lipid samples were repeatedly stored at –20 °C, freeze-thawed, and equilibrated, and new series of ^{2}H NMR spectra were recorded; practically identical spectra were recorded each time. Analytical TLC was used to determine if lipid degradation occurred during the NMR experiments; no significant degradation could be detected.

2.8 Data processing

The FIDs were converted into a suitable format and were fractionally left-shifted by the use of a home-written program FRLS. The FTNMR procedure consisted of the following steps: left-shifting was performed and the size of the files was set to 512 points, followed by zero-filling up to 4096 points. A line broadening of 50 Hz was applied. The membranes and the total lipid extracts contain a mixture of different lipids that give rise to a rather complex quadrupole splitting pattern. Therefore, in order to get a better spectral resolution, the spectra were de-Paked, a technique that offers a convenient way to resolve overlapping powder patterns (Bloom et al. 1981; Sternin et al. 1983). A FORTRAN program for iterative de-Paking in the frequency domain (Sternin 1982) was used. For each spectrum, 3 iterations of de-Paking were performed. The formation of the H_{II} phase was easily detected in the de-Paked spectra.

3 Results

In model membrane systems, the ^{2}H NMR spectra of phospholipids labelled in the C-2 position of both acyl chains are generally characterized by three quadrupole splittings (Seelig and Seelig 1980), due to the inequivalence of the two deuterons in the C-2 position of the *sn*-2 chain and to

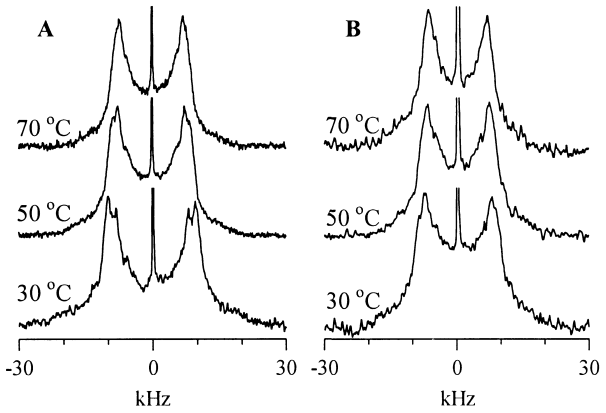

Fig. 1 A, B ^{2}H NMR spectra recorded at different temperatures from membrane samples prepared from *A. laidlawii* A-EF22. **A** Membrane sample from cells grown in medium supplemented with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2. **B** Pronase-treated membrane sample from cells grown in medium supplemented with 150 μM 18:1c-d_2

the different environments experienced by the *sn*-1 and *sn*-2 chains. The largest quadrupole splitting can be assigned to the *sn*-1 chain, while the other two splittings arise from the *sn*-2 chain. The ^{2}H NMR quadrupole splittings are reduced by approximately a factor of two when an L_α phase transforms into an H_{II} phase. This difference in the magnitude of the splittings between the two phases can be accounted for by the fact that there is an extra motional averaging in the H_{II} phase due to the translational diffusion around the symmetry axis of the water cylinders in this phase (Lindblom 1996).

3.1 Phase equilibria in membranes

Previous studies on the phase behaviour of *A. laidlawii* total lipid extracts hydrated with approximately 10 or 20% (w/w) water show that transitions from an L_α phase to an H_{II} phase, or an I_{II} phase, take place when the temperature is increased (Lindblom et al. 1986; Rilfors et al. 1994; Österberg et al. 1995). These observations turned our interest to investigate whether the lamellar-nonlamellar phase transition also can occur in intact membrane samples prepared from *A. laidlawii* cells.

Several samples were prepared from cells grown in media supplemented either with 150 μM 18:1c-d_2 or with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2. The ^{2}H NMR spectra obtained from these membrane samples showed only an L_α phase between 30 °C and 70 °C. The effect of higher temperatures was not investigated since the lipids could be expected to decompose. In order to exclude the possibility that this result is due to the fact that the lipid composition in the membranes is gradually adapted to the increasing temperature, the membrane-associated enzymes were inactivated in some samples (see Materials and methods) before the ^{2}H NMR spectra were recorded. However, no phase transition occurred between 30 °C and 70 °C in these membrane samples either (Fig. 1 A).

To examine if the water-exposed parts of the membrane proteins in the membrane samples inhibited the formation of an H_{II} phase by keeping the bilayers separated, most of the membrane proteins were removed through digestion with pronase. However, the lipids in the pronase-treated membrane sample formed only an L_α phase between 30 °C and 70 °C (Fig. 1 B).

The water contents in the samples with intact membranes and pronase-treated membranes were ~94% (w/w) and ~88% (w/w), respectively. The phase structure formed by membrane lipids often depends on the water content; this holds for isolated lipids as well as for total lipid extracts (Rilfors et al. 1984; Lindblom and Rilfors 1989). The absence of a phase transition in the membranes may depend on the high water content, and the phase equilibria for the total lipid extracts at increasing water contents were therefore investigated.

3.2 Effect of hydration on phase equilibria in total lipid extracts

In order to investigate the correlation between the water concentration and the formation of reversed nonlamellar phases in *A. laidlawii* total lipid extracts, the lipids were mixed with 20, 30, 40, 50 and 60% (w/w) ^1H_2O. Three total lipid extracts were examined. Moreover, the combined glucolipid and neutral lipid fractions were isolated from the total lipid extract containing 16:0-d_2 and 18:1c-d_2. The composition of these fractions, the relative amounts of the glucolipids and diacylglycerol (DAG) in the two total lipid extracts containing 18:1c-d_2, and the acyl chain compositions of the total lipid extracts are shown in Tables 1 and 2. The fractions of monoglucosyldiacylglycerol (MGlcDAG) and monoacyldiglucosyldiacylglycerol (MADGlcDAG) are decreased, and the fraction of diglucosyldiacylglycerol (DGlcDAG) is increased, when the degree of acyl chain unsaturation in the lipid extracts is increased. This is in line with previous results and has been interpreted to constitute a lipid regulation mechanism that strives to keep the balance between lamellar-forming and nonlamellar-forming lipids within a narrow range (Andersson et al. 1996, and references therein).

Table 1 The relative amounts (mol %) of diacylglycerol (DAG) and the glucolipids in total lipid extracts from *A. laidlawii* A-EF22

Lipid	Growth medium supplementation		
	75 μM 16:0-d_2 + 75 μM 18:1c-d_2	150 μM 18:1c-d_2 Batch 1	150 μM 18:1c-d_2 Batch2
DAG	1.2	1.2	0.5
MGlcDAG [a]	27.6	3.2	1.7
DGlcDAG [a]	53.3	85.2	85
MADGlcDAG [a]	17.9	10.5	12.8

[a] *MGlcDAG* Monoglucosyldiacylglycerol; *DGlcDAG* Diglucosyldiacylglycerol; *MADGlcDAG* Monoacyldiglucosyldiacylglycerol

Table 2 Acyl chain composition (mol %) in the total lipid extracts from *A. laidlawii* A-EF22. The lipid extracts contained the neutral lipids

Acyl chains [a]	Growth medium supplementation		
	75 μM 16:0-d_2 + 75 μM 18:1c-d_2	150 μM 18:1c-d_2 Batch 1	150 μM 18:1c-d_2 Batch 2
12:0	2.8	0.7	2.5
13:0	0.9	1	1.7
14:0	3.7	5.8	6.7
15:0	0.8	3.8	3.5
16:0	–	9.8	7.1
16:0-d_2	43.8	–	–
17:0	–	0.5	0.3
18:0	–	0.6	0.3
18:1c-d_2	47.5	75.9	76
unidentified	0.5	1.9	1.9

[a] The acyl chains are denoted as n : x were n is the number of carbons and x is the number of *cis* double bonds. The notation -d_2 indicates that the acyl chain contains 2 deuterium atoms in the α-position

Table 3 Effect of lipid hydration on the temperatures (T_{LH}) for the transition from a lamellar liquid crystalline (L_α) to a reversed hexagonal (H_{II}) phase in total lipid extracts isolated from *A. laidlawii* A-EF22. The lipid extracts contained the neutral lipids

^1H_2O % (w/w)	T_{LH} (°C) [a]		
	75 μM 16:0-d_2 + 75 μM 18:1c-d_2	150 μM 18:1c-d_2 Batch 1	150 μM 18:1c-d_2 Batch 2
20	43–46	30 [b]	30
30	53–57	50–55	50–55
40	–	60–65	n. i.
50	–	–	n. i.
60	–	n. i.	n. i.

[a] The upper limit of the temperature range investigated was 70 °C *n. i.*, not investigated
[b] Two lipid samples were examined

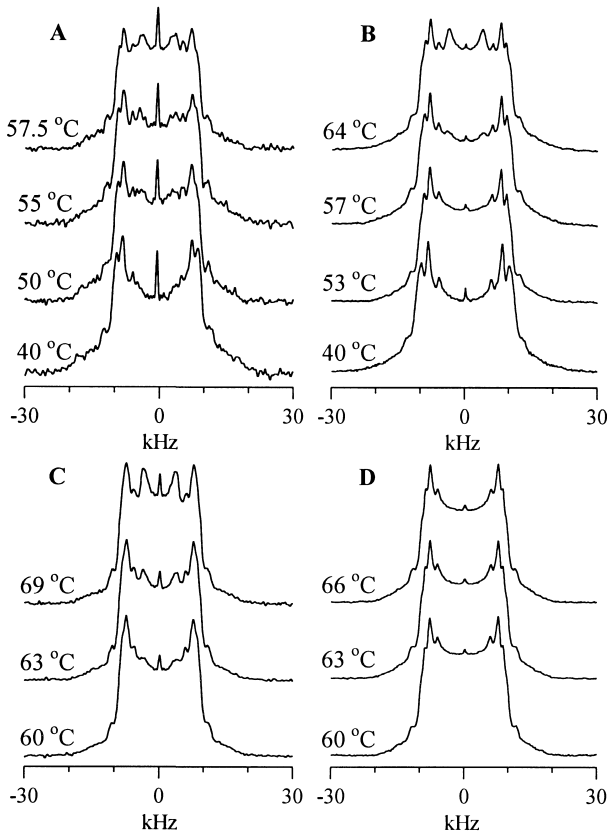

Fig. 2 A–D ^2H NMR spectra recorded at different temperatures from total lipid extracts isolated from *A. laidlawii* A-EF22; the lipid extracts contained the neutral lipids. The quadrupole splitting of about 9 kHz indicates the appearance of a reversed hexagonal phase in **A, B,** and **C. A** Total lipid extract containing 18:1c-d_2, 30% (w/w) ^1H_2O. **B** Total lipid extract containing 16:0-d_2 and 18:1c-d_2, 30% (w/w) ^1H_2O. **C** Total lipid extract containing 18:1c-d_2, 40% (w/w) ^1H_2O. **D** Total lipid extract containing 16:0-d_2 and 18:1c-d_2, 40% (w/w) ^1H_2O

In contrast to earlier studies of total lipid extracts from *A. laidlawii* the neutral lipid fractions were *not* removed. In studies of total lipid extracts from *E. coli* membranes (Morein et al. 1996) it was observed that the presence of the neutral lipid fraction decreased the temperature of the transition from an L_α phase to reversed nonlamellar phases (T_{NL}).

When the total lipid extracts were hydrated with 20% (w/w) ^1H_2O, an H_{II} phase appeared at approximately 30 °C and 43–46 °C in the lipid extracts derived from the cells grown in media supplemented with 150 μM 18:1c-d_2 and with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2, respectively (Table 3). Upon increasing the water concentration to 30% (w/w) the H_{II} phase started to form between 50–57 °C in all the extracts examined as judged from the appearing quadrupole splitting of about 9 kHz (Figs. 2 A and 2 B). With a water content of 40% (w/w), ^2H NMR spectra recorded from the total lipid extract derived from cells grown in the presence of 150 μM 18:1c-d_2 (batch 1) showed that an H_{II} phase started to form at 60–65 °C (Fig. 2 C). However, in the total lipid extract derived from cells grown in the presence of 75 μM 16:0-d_2 and 75 μM 18:1c-d_2, no indication of a transition to an H_{II} phase was detected up to 70 °C (Fig. 2 D). Only an L_α phase was formed up to 70 °C in the lipid extracts when the water concentrations were 50% (w/w) or larger (Table 3).

The combined glucolipid and neutral lipid fractions were isolated from the total lipid extract derived from cells grown in media supplemented with 75 μM 16:0-d_2 and 75 μM 18:1c-d_2 (Table 1). The phase behaviour of a sample consisting of these lipid fractions hydrated with 20% (w/w) of water was compared to that of the corresponding total lipid extract. The T_{LH} value was only 2–3 °C lower in the combined glucolipid and neutral lipid fractions (Fig. 3; Tables 3–5) despite the fact that the phospholipid fraction constituted about 34 mol% of the total lipid fraction.

The heterogeneity of the lipid head group composition and the different contributions from the α-deuterated *sn*-1 and *sn*-2 chains are factors that give rise to the many quadrupole splittings in the ^2H NMR spectra. The combined glucolipid and neutral lipid fractions gave rise to fewer quadrupole splittings than the corresponding total lipid ex-

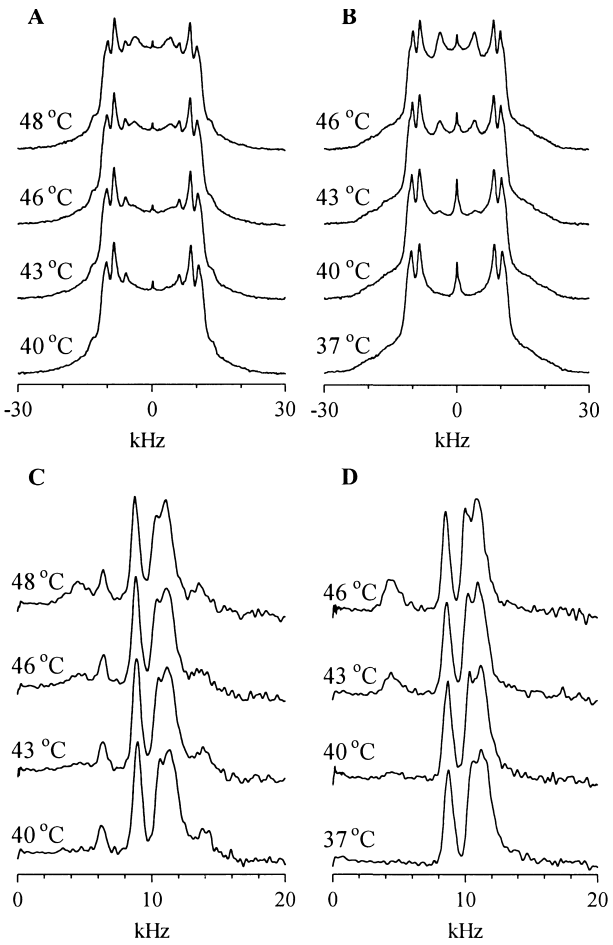

Fig. 3 A–D ^{2}H NMR spectra recorded at different temperatures from lipid extracts isolated from *A. laidlawii* A-EF22. The quadrupole splitting of about 9 kHz indicates the appearance of a reversed hexagonal phase. Note that only "half" the quadrupole splittings of the NMR spectrum is shown for the de-Paked spectra in **C** and **D.** **A** Total lipid extract with neutral lipids containing 16:0-d_2 and 18:1c-d_2, 20% (w/w) ^1H_2O. **B** The glucolipid and neutral lipid fractions containing 16:0-d_2 and 18:1c-d_2, 20% (w/w) ^1H_2O. **C** The de-Paked spectrum of **A;** 3 iterations of de-Paking were performed. **D** The de-Paked spectrum of **B**; 3 iterations of de-Paking were performed

tract (Figs. 3 C and 3D; Tables 4 and 5). The largest (~27 kHz) and the smallest (~12 kHz) splittings observed in spectra recorded from the total lipid extract are absent in spectra recorded from the combined glucolipid and neutral lipid fractions. Consequently, these splittings arise from the phospholipid fraction. This is in accordance with a previous investigation by Rance et al. (1983) in which *A. laidlawii* membrane lipids labelled with α-deuterated dihydrosterculic acid were studied.

3.3 Effect of divalent cations on the phase equilibria in total lipid extracts

The amounts of Mg^{2+} and Ca^{2+} ions present in *A. laidlawii* membranes in vivo have been estimated by Kahane et al. (1973). They determined the Mg^{2+} content to be 1.55 µg per mg of membrane protein and that 60 to 83% was bound to the lipid fraction. However, they could only detect small amounts of Ca^{2+}. From these data it can be estimated that the amount of Mg^{2+} is in the range of 0.9–1.2 µg per mg lipid if it is assumed that the membrane is composed of equal amounts of protein and lipid by weight (Wieslander and Rilfors 1977).

Mg^{2+} or Ca^{2+} was included in the water added to the total lipid extracts prepared from the cells grown in the medium supplemented with 150 µM 18:1c-d_2. The molar ratio cations : anionic phospholipids was 1/10; in the case of Mg^{2+} this corresponds to 0.8 µg of ions per mg of lipid, which is in the same range as the amount found in vivo. The water concentration in the samples was chosen to be 20% (w/w). The phase behaviour was compared to that of a reference without cations, prepared from the same batch of total lipid extract. The temperature at which an H_{II} phase started to form was approximately 30 °C in all three samples and no significant differences between the samples were observed at higher temperatures.

Table 4 Quadrupole splittings (Δ^2H) obtained at different temperatures in ^{2}H NMR spectra recorded from a total lipid extract containing α-deuterated 16:0 and 18:1c. The lipids were hydrated with 20% (w/w) ^1H_2O

Temperature (°C)	Δ^2H (kHz)						
40		12.2	17.6	21.1	22.3	27.1	28.1
43		12.4	17.5	20.9	22.1	27.3	
46	9.1 [a]	12.5	17.4	20.6	22	27	
48.5	8.9	12.5	17.3	20.3	21.8	26.9	

[a] This quadrupole splitting indicates the appearance of a reversed hexagonal phase

Table 5 Quadrupole splittings (Δ^2H) obtained at different temperatures in ^{2}H NMR spectra recorded from the combined glucolipid and neutral lipid fractions containing α-deuterated 16:0 and 18:1c. The lipids were hydrated with 20% (w/w) ^1H_2O

Temperature (°C)	Δ^2H (kHz)			
37		17.4	21.1	22.3
40		17.3	20.6	22.3
43	8.7 [a]	17.2	20.3	21.8
46	8.5	17	20.1	21.8

[a] This quadrupole splitting indicates the appearance of a reversed hexagonal phase

4 Discussion

Experiments have been performed previously on *A. laidlawii* membranes with various techniques (see e. g. Casal et al. 1980; Rance et al. 1980; Silvius et al. 1980; Moore et al. 1993). However, the main purpose of those experiments was to study the lamellar gel (L_β) to L_α phase

transition temperature for cells, membranes, and the extracted total lipids. One aim of the present study was to establish whether the lipids in intact *A. laidlawii* membranes can form nonbilayer structures. ^{2}H NMR spectra recorded from membranes labelled with α-deuterated fatty acids show that only an L_α phase is formed between 30 and 70 °C. The reorganization of an L_α phase to an H_{II} phase has been shown both theoretically and experimentally to be initiated by a close proximity of the lipid bilayers (Borovjagin et al. 1982; Siegel 1986 a, 1986 b), and it has been proposed that the apposed bilayers must be closer than 1 nm for the phase transition to be initiated. A large fraction of the proteins in the *A. laidlawii* membrane was therefore removed by digestion with pronase. However, no phase transition could be detected between 30 and 70 °C.

Total lipid extracts from *A. laidlawii* membranes formed an H_{II} phase up to a water content of 40% (w/w). One can speculate about the reason for the difference in the transition temperature between the lipid extracts when the water content is 20% (w/w) (Table 3). Previous investigations of the phase equilibria of total lipid extracts isolated from maize chloroplasts and *E. coli* revealed that the T_{NL} values were less reproducible when the neutral lipid fraction was still present in the lipid extracts (Rivas and Luzzati 1969; Morein et al. 1996). The difference in the temperature (T_{LH}) for the L_α to H_{II} phase transition observed in the present study when the water content is 20% (w/w) may therefore be the result of a natural variation that emerges when the samples are prepared from living cells (Rivas and Luzzati 1969).

All the total lipid extracts formed only an L_α phase when the water content was 50% (w/w) or larger. This finding is in accordance with earlier reports stating that a high degree of hydration often favours the formation of an L_α phase in various lipid-water systems (Luzzati and Husson 1962; Gulik-Krzywicki et al. 1967; Rivas and Luzzati 1969; Rilfors et al. 1984; Seddon 1990). It is therefore concluded that the high water concentrations (88% (w/w) or larger) in the membrane samples prevent the formation of nonlamellar phases irrespective of the presence and the structure of the membrane proteins.

Since the combined glucolipid and neutral lipid fractions and the corresponding total lipid extract have similar T_{LH} values (Fig. 3; Tables 3–5), the phospholipids apparently have a very modest influence on the T_{LH} value of a total lipid extract. This conclusion is surprising since the addition of anionic phospholipids to an H_{II} phase generally raises the T_{LH} value (Rilfors et al. 1984). The result may therefore indicate that hydrogen bonding occurs between the polar head groups of the phospholipids and the glucolipids. A recent monolayer study of individual lipids and total lipid extracts from *A. laidlawii* supports this suggestion (Andersson et al. 1997).

The addition of physiologically relevant concentrations of Ca^{2+} and Mg^{2+} ions to total lipid extracts did not affect the phase equilibria significantly. Earlier investigations have shown that Mg^{2+} and Ca^{2+} can affect the phase behaviour of anionic phospholipids. For example, these ions induce the formation of an H_{II} phase when mixed with diphosphatidylglycerol (Rand and Sengupta 1972; Killian et al. 1994) or phosphatidic acid (Verkleij et al. 1982), while they stabilize an L_β phase when mixed with phosphatidylglycerol or phosphatidylserine (Verkleij et al. 1974; Hauser and Shipley 1984). The anionic lipids occurring in the *A. laidlawii* A-EF22 membrane are phosphatidylglycerol and two glycerophosphoryl derivatives of DGlcDAG (Wieslander and Rilfors 1977; Hauksson et al. 1994). One of the glycerophosphoryl derivatives forms long thread-like micelles in a highly diluted water solution (Danino et al. 1997) and the probability that this lipid forms an H_{II} phase in the presence of divalent cations is therefore small.

Investigations of the phase behaviour of total lipid extracts (including the neutral lipid fraction) prepared from *E. coli* cells grown at 37 °C showed that a total lipid extract hydrated with 70% (w/w) of water started to form an isotropic phase between 50 and 60 °C; this transition temperature was decreased about 10 °C if the lipids were extracted from cells grown at 17 or 27 °C (Morein et al. 1996). These results thus differ markedly from those obtained with total lipid extracts isolated from *A. laidlawii*, since no nonlamellar phases could be induced in these extracts below 70 °C with water contents of 50% (w/w) or higher. This difference might be explained by the different structures of the cell envelope in the two bacterial species. *A. laidlawii* cells are surrounded only by a single membrane, the plasma membrane, while *E. coli* cells are surrounded by an inner and an outer membrane separated by a cell wall. It is crucial for survival of a cell to maintain the membrane lipids in a bilayer structure at the growth temperature. An *E. coli* cell might be able to deal with a lipid composition that has a greater tendency to form nonlamellar structures, since the firm cell wall can counteract such structural tendencies by preventing the two lipid monolayers to curl up. It might even be necessary for *E. coli* cells to have a pronounced tendency to form nonbilayer structures, since this could enable the cells to form adhesion sites (Oliver 1996) between the two membranes.

The phase equilibria of a total lipid extract from another Gram-negative bacterium, *Pseudomonas fluorescens*, have also been studied (Mariani et al. 1990). An I_{II} phase is straddled by an H_{II} phase in the phase diagram, and for lipids with 50% (w/w) of water the H_{II} phase forms just 5 °C above the growth temperature of the cells. A comparison can also be made with the phase equilibria of a total lipid extract from the archaebacterium *Sulfolobus solfataricus* (Gulik et al. 1985). Cells belonging to the domain *Archaea* are surrounded by a cell wall, although of another chemical composition than the cell wall of Gram-negative and Gram-positive bacteria. The total lipid extract from *S. solfataricus* forms two different I_{II} phases; one of the phases is formed at the growth temperature of the cells when the lipids are hydrated with 45–50% (w/w) of water. Thus, also *P. fluorescens* and *S. solfataricus* have membrane lipids that are more prone to form reversed nonlamellar phases than the lipids of *A. laidlawii*. Finally, total lipid extracts isolated from the organelles mitochondria and chloroplasts form only an L_α phase above water contents of approxi-

mately 20% (w/w) (Gulik-Krzywicki et al. 1967; Rivas and Luzzati 1969). The different abilities of the total lipid extracts to form reversed nonlamellar phases are probably not due to large variations in the degrees of maximum hydration of the lipid extracts, since the presence of 3% (w/w) of ionic lipids in an L_α phase already results in the uptake of very large amounts of water (Gulik-Krzywicki et al. 1969). With this limited set of data as basis it can therefore be speculated that cell wall-surrounded cells can tolerate a membrane lipid composition that has a rather marked ability to form reversed nonlamellar phases; it may even be a functional advantage for these cells to have such a lipid composition. The reason why this is important for the cell has been subjected to many recent suggestions and speculations in the literature, and the role played by such "lipid forces" is currently investigated in several laboratories (Epand 1996; Marsh 1996, Thurmond and Lindblom 1997).

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